

Identification of the High Affinity Receptor Binding Region in Human Immunoglobulin E*

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We have investigated the capacity of N- and C-terminally truncated and chimeric human (h) IgE-derived peptides to inhibit the binding of ¹²⁵I-labeled hIgE, and to engage cell lines expressing high and low affinity receptors (FcεRI/II). The peptide sequence Pro³⁴³–Ser³⁵³ of the hCε3 domain is common to all hε-chain peptides that recognize hFcεRI. This region in IgE is homologous to the A loop in Cγ2 that engages the rat neonatal IgG receptor. Optimum FcεRI occupancy by hIgE occurs at pH 6.4, with a second peak at 7.4. N- or C-terminal truncation has little effect on the association rate of the ligands with this receptor. Dissociation markedly increases following C-terminal deletion, and hFcεRI occupancy at pH 6.4 is diminished. His residue(s) in the C-terminal region of the ε-chain may thus contribute to the high affinity of interaction. Grafting the homologous rat ε-chain sequence into hIgE maintains hFcεRI interaction without conferring binding to rat FcεRI. hFcεRII interaction is lost, suggesting that these residues also contribute to hFcεRII binding. hε-chain peptides comprising only this sequence do not block hIgE/hFcεRI interaction or engage the receptor. Therefore, sequences N- or C-terminal to this core peptide provide structures necessary for receptor recognition.

Antibodies of the immunoglobulin (Ig)E isotype sensitize target cells expressing the class-specific Fc receptors for antigen-induced mediator release, by binding through residues located in the Fc portion of the molecule (1, 2). The potent pharmacologically active substances that are released in response to this stimulus cause the clinical symptoms of allergy.

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Strategies that block the initial sensitization of target cells with antigen-specific IgE have been explored following the demonstration that human (h)¹ myeloma IgE-derived Fcε fragments generated by proteolytic cleavage with papain (1, 2), which produces peptides comprising hε-chain residues 1–226 and 227–547, can competitively inhibit the binding of IgE to cells expressing high affinity receptor (FcεRI) (1, 2), whereas cleavage products of pepsin digestion, which generates ε fragments spanning residues 1–338, 339–349, and 350–547 (3) do not inhibit binding. This observation initiated the quest for progressively smaller peptides as potential IgE antagonists (reviewed in Ref. 4). In early studies, the inhibition of passive cutaneous anaphylaxis in human skin was used to assess the FcεRI-blocking activity of proteolytic fragments or recombinant IgE-derived peptides expressed in *Escherichia coli* (1, 5, 6). This led to the proposal that sequences N- and C-terminal to Val³³⁶ contribute structures necessary for FcεRI interaction (6). More recent studies aimed at the identification of the receptor binding site(s) employed chimeric human/mouse IgE antibodies, ε/γ chimeras, site-specific mutagenesis, anti-IgE antibodies, or IgE-derived peptides (7–15). They indicate that the site(s) in IgE that interact(s) with the Fc receptors depend(s) on structures associated with residues located in the Cε3 domain, although Cε4 involvement has also been invoked (11, 12). Furthermore, it has been suggested that IgE/FcεRI interaction is mediated primarily by electrostatic interactions (14) and dependent on the entire Cε3 in its native conformation (10), while the Cε4 domains are essential for the maintenance of the active conformation of the Cε3 domain (7, 16). Our earlier investigations showed that while IgE/FcεRII interaction is critically dependent on Cε4 or its homologue Cγ3 (16), it is possible to delete the entire Cε4 domain and more than 60% of residues in Cε3 and still maintain the FcεRI-blocking capacity of the recombinant ε-chain fragment (6, 17).

Based on our demonstration of the parallel nature of the inter-ε-chain disulfide bonds in hIgE (18), we developed a structural model that predicts that an exposed and probably flexible segment connects the globular portions of the Cε2 and Cε3 domains (18, 19). Subsequently, Gould *et al.* (20, 21) claimed that the N-terminal 11 residues in Cε3, which are included in this segment, are essential for FcεRI binding. This proposal relied on studies where the biological activity of recombinant ε-chain fragments was tested by blocking the binding of ragweed-specific IgE to mast cells in the skin of the senior investigator conducting the study (6). When the fallibility of the passive cutaneous anaphylaxis reaction in assessing the biological activity of recombinant IgE-derived fragments emerged

¹ The abbreviations used are: h, human; r, rat; GST, glutathione S-transferase; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin.

(17, 22), we re-assessed the biological activity of these and additional truncated fragments using our recently developed receptor binding assay, which allowed us to study direct binding of IgE-derived ligands to rat (r) basophilic leukemia cells (RBL-2/2/C) transfected with the α-chain of hFcεRI (23).

In the present investigation we describe the capacity of a series of overlapping N- and C-terminally truncated and chimeric ε/γ-chain derived fragments, expressed as glutathione S-transferase (GST) fusion proteins in *E. coli* to bind directly to and block the binding of hIgE to RBL-2/2/C cells. We show that the peptide sequence spanning amino acid residues Pro³⁴³–Ser³⁵³ is common to all recombinant ε-chain fragments capable of binding to FcεRI. Deletion of this sequence is associated with a complete loss of receptor recognition, confirming earlier observation by others that grafting the homologous sequence from IgG1 into hIgE reduces FcεRI binding by 97% (14). Replacing this sequence in hIgE by the homologous rat sequence maintains binding to hFcεRI, but there is a loss of hFcεRII interaction, confirming earlier observations by others that rodent IgE recognizes only hFcεRI but not hFcεRII (10). Since recombinant GST-ε-chain fusion proteins containing this sequence do not block IgE/FcεRIα interaction, we conclude that sequences N- or C-terminal to this core peptide are essential for the provision of additional structural scaffolding in order to generate a receptor binding conformation. Viewed in the context of our model structure for IgE (18, 19), this core peptide has been computed to form a loop proximal to the interface between the Cε3/4 domains that is homologous to the site in rodent IgG involved in the binding to the groove formed by the α1 and α2 domains of the neonatal FcγRn (24). Interestingly, as for IgG/FcγRn interaction, we also observe two pH optima at pH ~6.4 and 7.4 for hIgE/FcεRIα interaction. While N- or C-terminal truncation has little effect on the association rate, deletion of C-terminal sequences increases the rate of dissociation several hundred-fold and reduces receptor occupancy at pH 6.4. The slow dissociation of IgE from FcεRIα therefore may be due, at least in part, to the stabilization of the interaction by His residues in the C-terminal region of the ligand.

EXPERIMENTAL PROCEDURES

Gene Constructs and Site-specific Mutagenesis—The numbering scheme for hε-chain amino acid residues used in previous publications (6, 16–18) has been maintained. Polymerase chain reaction (PCR) was used to amplify ε-chain fragments comprising the entire Fc region (residues 226–547), the Cγ3 from mouse IgG2a, the Cε2 domain (residues 226–329), and the Cε4 domain (residues 440–547). N-terminal deletions of the Fc region were prepared starting at amino acid residue positions 326, 330, 340, 342, 343, 344, 345, 350, and 355 and terminating at residue 547. C-terminal deletions were prepared starting at amino acid residue 226 and terminating at residues 361, 357, 354, 353, 352, 345, and 340. The DNA products were purified by agarose gel electrophoresis, digested with appropriate restriction enzymes, and subcloned into the bacterial expression plasmids pGEX-3X and pGEX-KG, which direct the synthesis of foreign polypeptides in *E. coli* as fusions with the 26-kDa GST (26). Cloning the recombinant ε-chain fragments in frame to the 3' end of the GST gene facilitates the production of large amounts of fusion protein (~500 mg/liter). In addition to a versatile multiple cloning site, the vectors have been engineered so that the GST carrier can be cleaved off by digestion with coagulation factor Xa or thrombin. The initial screening for receptor-blocking activity was carried out with partially purified GST-ε-chain fusion peptides. Following affinity purification on rabbit anti-GST affinity columns and GST removal with thrombin, ε-chains showed identical receptor-blocking capacities when compared with GST-ε-chain fusion peptides. Therefore, this step was eliminated, and all assays described in this study were carried out with affinity-purified GST-ε-chain fusion peptides. Short GST fusion peptides comprising ε-chain residues 338–359 and 340–357 were also generated. Site-specific mutagenesis was performed by overlap extension PCR (25). Bacterial strains used as host for transformation were JM109 or MC1061.

For the construction of the chimeric h/r IgE molecule we employed

the ε-chain expression plasmids pSV-V_{NP}h/r_ε (18). A construct where the sequences known to be essential for hFcεRI interaction had been replaced by the homologous rat sequence encoding residues 341–356 was also generated by overlap extension PCR (25). The template for PCR was a 3.4-kilobase pair IgE Cε1–4 genomic DNA cassette cloned into the *Bam*HI site in pUC19 (pHe). A 719-base pair fragment coding essentially for Cε2–3 was generated by PCR.

This involved two rounds of PCR and four primers, two external (5' *Bgl*II, CGTGAAGATCTTACAGTCGTC; 3' *Nco*I, CCTGCCCATGGCT-CACCG) and two internal primers (5' h-r16, CCTCGACCTGTATGAA-AATGGGACTCCCAAACTTACCTGTCTGGTGGTGGACCTG; 3' h-r16, CCATTTTCATACAGGTCGAGGGGACTGGGTGGGATTAGGTAGG-CGCTACCCCTCT).

For each PCR, reaction mixtures contained 200 ng of template, 2 μg of each primer, 1 mM dNTPs, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂ in 100 μl, and following a hot start 1 unit of *Taq* polymerase was added. An initial denaturation cycle at 96 °C for 6 min, 64 °C for 2 min, 72 °C for 1 min 30 s was followed by 30 cycles at 94 °C for 1 min 30 s, 64 °C for 1 min 30 s, 72 °C for 1 min 30 s. The resultant 719-base pair fragment was cloned into pHe using *Bgl*II and *Nco*I sites to give a chimeric Cε1–4 cassette, which was subcloned, using the *Bam*HI sites, into the mammalian expression vector pSV-V_{NP} (18). The orientation of this cassette was checked by PCR.

The identity of all gene constructs was confirmed by sequencing the DNA of both strands.

Gene Expression—*E. coli* strains transformed with the expression plasmids were grown overnight at 37 °C, and the overnight culture was diluted 100-fold into LB broth containing 100 μg/ml ampicillin and grown to an absorbance of 0.4 at 600 nm at 37 °C. The inducer isopropyl-1-thio-β-D-galactopyranoside (Sigma) was added to a final concentration of 0.1 mM, and the cultures were grown under constant shaking at 37 °C for 4 h. Bacterial cells were harvested by centrifugation at 5,000 × *g* for 15 min, and the pellets were frozen at –70 °C until purification of the recombinant proteins. Freezing and subsequent thawing of the bacterial pellets were essential to obtain effective solubilization of the recombinant proteins, which are expressed as insoluble inclusion bodies.

The chimeric pSV-V_{NP} construct was linearized using *Pvu*I and electroporated into the J558L plasmacytoma cell line (18). J558L cells were cultured in Dulbecco's modified Eagle's medium (10% fetal calf serum, penicillin/streptomycin, gentamicin) and selection medium (Dulbecco's modified Eagle's medium, 10% fetal calf serum, penicillin/streptomycin, gentamicin, mycophenolic acid, xanthine, and hypoxanthine) was added 48 h after electroporation (18). High secreting clones were selected by enzyme-linked immunosorbent assay.

Purification of Recombinant GST-ε-chain Fusion Proteins from *E. coli* Cell Pellets—This was carried out using procedures described for the purification of recombinant ε-chain fragments expressed in *E. coli* (6). Frozen cell pellets were defrosted on ice before homogenization (5-fold pellet volume) in 0.05 M Tris-HCl buffer, pH 7.9, containing 2 mM EDTA, 0.1 mM dithiothreitol, 1 mM β-mercaptoethanol, 0.25 M NaCl, 0.1% sodium deoxycholate, 25 μg/ml phenylmethylsulfonyl fluoride, 5% glycerol. The homogenates were dispersed by sonication before the addition of 100 μg/ml lysozyme and 20 μg/ml DNase I. Homogenates were kept on a rotary shaker for 12–15 h at 4 °C before centrifugation at 10,000 × *g* for 10 min. The pellets were washed twice in a 20-fold pellet volume of 0.05 M Tris-HCl buffer, pH 7.9, containing 1 mM EDTA, 0.1 M NaCl, 25 μg/ml phenylmethylsulfonyl fluoride. Inclusion bodies from cell pellets were solubilized in 0.05 M Tris-HCl buffer, pH 7.9, containing 8 M urea, 1 mM EDTA, 0.1 M NaCl, 25 μg/ml phenylmethylsulfonyl fluoride and dialyzed for 12 h against a 200-fold volume of the same buffer omitting urea but with the addition of 0.1 mM dithiothreitol and 1 mM β-mercaptoethanol. Insoluble materials were removed by centrifugation, and 30–75% of recombinant ε-chain peptides were found in the supernatant fraction. Affinity purification from this fraction was carried out using a rabbit anti-GST antiserum coupled to Sepharose 4B. The chimeric h/r antibody was purified from cell culture supernatants using NP-specific affinity columns and analyzed by polyacrylamide gel electrophoresis (PAGE) and immunoblotting (6, 18).

Gel Electrophoresis and Immunoblotting—PAGE and electroblotting procedures have been described before (6, 18). Blots were developed with a polyclonal horseradish peroxidase-conjugated anti-hIgE antibody (Dako).

Ligand Binding Studies and Cell Culture—hIgE V_{NP} (18) was iodinated as described previously, and the conditions for ligand binding and cell culture have been published (23). Affinity-purified GST-ε-chain fusion peptides were iodinated at 0–4 °C in 0.4 M phosphate pH 7.4/7.5 using 4.4 μCi of Na¹²⁵I and 150–300 μg of peptide in tubes coated with

40 μg of IODO-GEN (Pierce). Following a 15-min incubation period, the reactions were terminated by removing fluid from the coated tubes. Each preparation was fractionated on a 140-ml Sephacryl S-200 column (Pharmacia), pre-equilibrated with binding buffer (phosphate-buffered saline, 0.2% BSA, pH 7.4), which effects the separation of dimers and monomers. Following γ-counting of collected fractions, peak fractions were pooled, aliquoted, and stored at -70 °C. Specific activity ranged from 5.8–15.5 μCi/μg.

The conditions for maintenance of RBL-2H3 cell lines transfected with the α-chain of hFcεRI (RBL-2/2/C) have been described (23). RBL-2/2/C clones were plated into 48-well plates at an initial plating density of 10⁵ cells/well and incubated with 10⁻⁶ M dexamethazone for 24 h at 37 °C. In preliminary experiments, RBL-2/2/C cells were incubated with increasing concentrations of ¹²⁵I-labeled ligands (0.1–7.5 μg/ml) to determine the minimum saturation concentrations for FcεRI binding. The proportion of molecules capable of binding to FcεRI was 78–91% for ¹²⁵I-labeled h and h/r IgE V_{NP}, while the bindable portion of the ¹²⁵I-labeled GST-ε(226–547), GST-ε(326–547), GST-ε(340–547), and GST-ε(226–354) was 41, 56, 53, and 27%, respectively. The number of rFcε molecules bound per cell was calculated on the basis that GST-ε(326–547) and GST-ε(340–547) are dimers, while GST-ε(226–354) is a monomer.

Nonspecific binding was determined using a 50–100-fold molar excess of nonlabeled hIgE over ¹²⁵I-hIgE, and the same amount of GST was used as a negative control. The binding of recombinant proteins to FcεRIα was determined indirectly, after correcting for nonspecific binding (7–17%), by calculating the percentage of inhibition of ¹²⁵I-IgE binding to cells. To measure the inhibition (IC₅₀) of ¹²⁵I-IgE binding to RBL-2/2/C or the 8866 lymphoblastoid cell line (18) by native and chimeric h/r IgE V_{NP} and recombinant ε-chain fragments, cells were preincubated with increasing concentrations (10⁻¹²–10⁻⁵ M) of each of the unlabeled peptides in 125 μl of binding buffer or, as control, binding buffer alone at 22 °C for 1 h, before the addition of 50 μl of binding buffer containing ¹²⁵I-hIgE (1 nM). After 45 min, the cells were washed twice with 0.5 ml of ice-cold binding buffer and lysed with 0.5 ml of lysis buffer (0.5 M NaOH, 1% Triton X-100). Samples (0.25 ml) were removed and counted for 5 min on a LKB1277 γ-counter.

The kinetics of association between RBL-2/2/C cells and ¹²⁵I-labeled h and h/r chimera, GST-ε(226–547), GST-ε(326–547), GST-ε(340–547) (2 μg/ml, in binding buffer), and GST-ε(226–354) (0.7 μg/ml, in binding buffer) were measured at 22 °C during the first 300 s of incubation at pH 7.4. Rate constants were calculated on the basis that GST-ε(226–547), GST-ε(326–547), and GST-ε(340–547) are dimers, while GST-ε(226–354) is a monomer. The forward rate constant (k_{+1}) was calculated as $V_0/C_0 \times R_0$, where V_0 represents the initial rate of binding, and C_0 and R_0 represent the concentration of ligand and receptor number (~130,000 α-chains/cell (23)). To determine the dissociation rate constant (k_{-1}), cells were preincubated for 1 h at 22 °C with 125 μl of the following ¹²⁵I-labeled ligands: h and rat IgE, the h/r chimera, GST-ε(226–547), GST-ε(326–547), GST-ε(340–547), and GST-ε(226–354/7) (ligand concentration as for the determination of k_{+1}). Cells were washed twice with 0.5 ml of binding buffer before 125 μl binding buffer containing a 50-fold molar excess of unlabeled hIgE or binding buffer was added. At t_0 and after 15-, 30-, 60-, 120-, and 180-min intervals, the cells were washed twice with 0.5 ml of ice-cold binding buffer, solubilized in 0.5 ml of lysis buffer (0.5 M NaOH, 1% Triton X-100), and 0.25-ml samples were assayed for cell-bound ¹²⁵I.

The pH optimum for the binding of ¹²⁵I-labeled hIgE and the GST-ε-chain fragments to FcεRI was determined by incubating RBL-2/2/C cells (23) in 48-well plates with 100 μl of 50 mM phosphate-buffered saline containing 0.2% BSA (pH range 5.9–8.1) for 10 min at 37 °C before adding 50 μl of 2 μg/ml ¹²⁵I-hIgE or 0.7 μg/ml of the ¹²⁵I-GST-ε-chain fragments. Cells were incubated for 30 min, and the excess protein was removed by washing with saline containing 0.2% BSA before measurement of cell-bound label. Results were corrected for nonspecific binding.

Potassium Iodide Titrations—The generation of IgE V_{NP}hε-(Cys³²⁸ → Met) has been described (18). The conformations of native and mutant (Cys³²⁸ → Met) recombinant IgE were investigated by comparing their intrinsic fluorescence. Solute quenching of protein fluorescence involved excitation of Trp residues at 297 nm and measurement of emission in the range 300–450 nm. Potassium iodide was added gradually to give a quench profile for each protein. Mathematical analysis was carried out according to the Stern-Volmer law (28).

RESULTS AND DISCUSSION

In the present study, we focused on the identification of the site(s) that determine the interaction of hIgE with its cellular receptors. The strategies employed for the expression of an overlapping family of chimeric GST-ε-chain fusion proteins are outlined in Fig. 1. *Panel A* summarizes the receptor-binding capacities of the GST-ε-chain fusion proteins, that of a chimeric ε/γ peptide, and that of a chimeric h/r IgE molecule. The assignment of biological activities is based on (i) competition and (ii) direct binding studies detailed in Fig. 2 and Table I. In Fig. 1, *panels C* and *D* show the electrophoretic mobilities of C- and N-terminally truncated recombinant GST-ε-chain fusion proteins immunoprecipitated with a rabbit anti-GST antiserum, followed by PAGE analysis under nonreducing conditions and immunoblotting with a horseradish peroxidase-labeled rabbit anti-IgE serum. As shown in Fig. 1, C-terminal truncation yields a number of ε-chain peptides for each construct. As judged by PAGE (Fig. 1C) and column chromatography (data not shown), approximately one-third of the peptides in each set corresponds to the full-length fusion peptide as a monomeric fragment. None of these fragments show any propensity to dimerize, although biologically inactive polymeric aggregates form at concentrations >1.3 mg/ml. A set of identical fragments is observed following analysis under reducing conditions (data not shown). Most of the smaller ε-chain fragments represent proteolytic cleavage fragments that are recognized by monoclonal antibodies specific for the Cε2 domain.² In contrast, deletion of N-terminal sequences gives rise to two ε-chain fragments and their apparent molecular weight under nonreducing (Fig. 1D) and reducing conditions (data not shown) indicates that they correspond, in almost equal quantities, to monomeric and dimeric GST-ε-chain fusion proteins.

The present investigation confirms our previous observations, which show that only those peptides that contain Cε4 or the homologous Cγ3 domain can engage both FcεRI and FcεRII (6, 16), while C-terminal truncation of the ε-chain results in elimination of binding to FcεRII. As summarized in Fig. 1A, sequences common to all fragments capable of binding to FcεRIα comprise residues Pro³⁴³–Ser³⁵³ in the Cε3 domain. Further deletion from either the C- or N-terminal end beyond these residues is associated with a loss of FcεRI binding. As shown in Fig. 2 and Table I, GST-ε(340–547) and GST-ε(226–354), which comprise the core peptide, inhibit the binding of hIgE with an IC₅₀ in the nanomolar range. In contrast, blocking of IgE/FcεRI interaction by the GST control, GST-ε(226–340), GST-ε(355–547), and GST-ε(440–547) is identical and cannot be detected even above micromolar concentrations. These results confirm observation by others who find that recombinant IgE-derived fragments comprising residues 355–547 do not block hIgE binding to hFcεRI (11) and that substitution of residues 346–353 by the homologous sequence from IgG1 reduces binding of the chimera to background levels (14).

As shown in our model structure of hIgE-Fc (Fig. 3) (18), this sequence forms a loop that is homologous to the loop in rIgG shown to bind to the neonatal FcγRn (24). A further similarity emerged when we investigated the pH dependence of the binding of hIgE to FcεRI. As shown in Fig. 4 two pH optima are observed for the binding of hIgE to FcεRI, and occupancy of the receptor is almost twice as high at pH 6.4 as at pH 7.4. Although the significance of this is not known, it is tempting to speculate that hIgE has evolved the lower pH optimum as a result of its physiological importance in the fight against parasitic infestations in the lumen of the intestine at acid pH.

Data summarized in Table I show that N- or C-terminal

² B. Helm, unpublished observations.

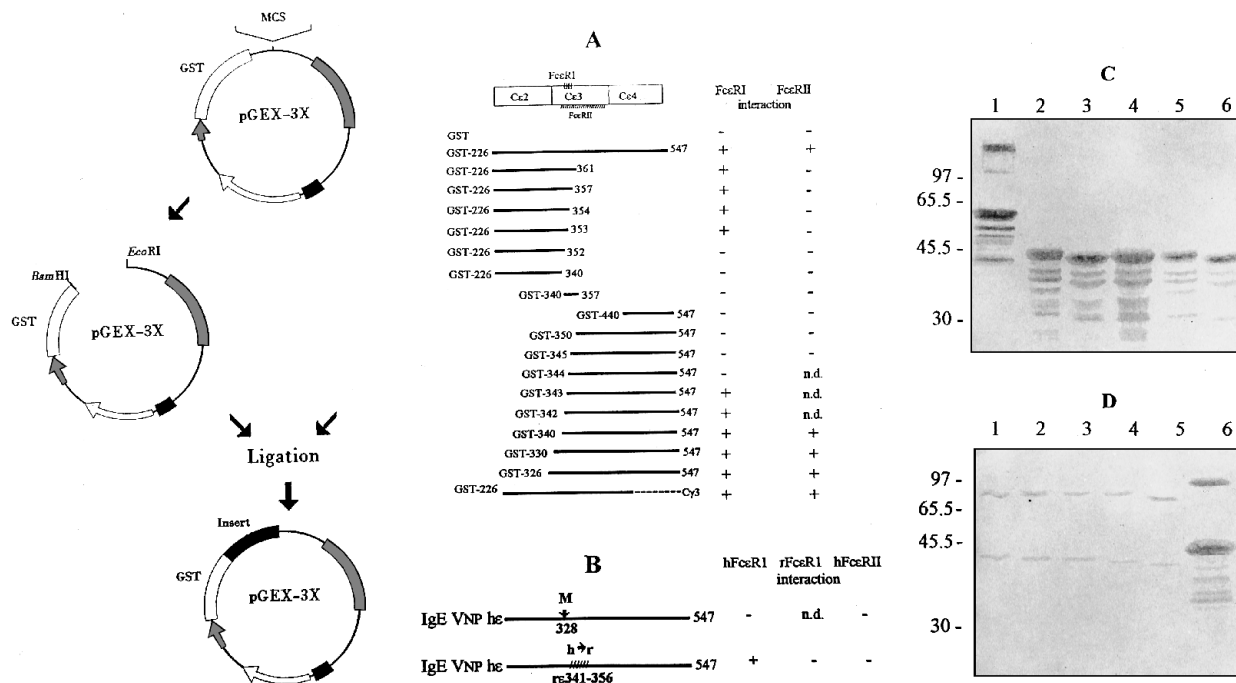


FIG. 1. Gene constructs, expression products, and mapping of receptor binding regions in human IgE. Recombinant ϵ -chain gene fragments were subcloned into the multiple cloning site (MCS) of the bacterial expression plasmids pGEX-3X and pGEX-KG (25) and expressed in *E. coli* (6, 18). The ϵ -chain expression plasmids pSV-V_{NP}he/r ϵ were employed for the construction of mutant and chimeric IgE molecules and expressed in the J558L myeloma cell line (18). **Panel A** and **B** summarize the ability of the truncated, chimeric, and mutant ϵ -chain variants to bind to FcεRIα expressed on RBL-2H3.1 and RBL-2/2C cells (23, 27) and to FcεRII expressed on the 8866 lymphoblastoid cell line (18). Initial screening for biological activity was determined by assessing the capacity of GST- ϵ -chain fusion proteins to inhibit the binding of ¹²⁵I-labeled hIgE (1 nM) to the receptors. The degree of inhibition effected by nonbinders was identical, within limits of experimental error, to that observed with GST, which was included as a negative control (see Fig. 2). Purification of truncated recombinant GST- ϵ -chain fusion proteins and mutant and chimeric IgE molecules was carried out as described (see "Experimental Procedures"). Ligands were labeled with ¹²⁵I for direct binding studies (see Table I). Nonbinders showed no binding above background even at concentrations above 10⁻⁵ M. **Panel C** and **D** show GST- ϵ -chain fusion proteins that were immunoprecipitated with a rabbit anti-GST serum, followed by SDS-PAGE (12%) separation under nonreducing conditions and immunoblotting with a horseradish peroxidase-labeled rabbit anti-human IgE serum. **Panel C**, lanes 1–6, GST- ϵ (226–547), GST- ϵ (226–361), GST- ϵ (226–357), GST- ϵ (226–354), GST- ϵ (226–352), GST- ϵ (226–340). **Panel D**, lanes 1–6, GST- ϵ (326–547), GST- ϵ (340–547), GST- ϵ (343–547), GST- ϵ (345–547), GST- ϵ (350–547), GST- ϵ (226–440)-Cys3. +, receptor binding; –, loss of receptor binding; n.d., not determined.

truncation has a negligible effect on the rate of association of biologically active ϵ -chain fragments with FcεRI. In contrast, the rate of dissociation increases several hundred-fold following the deletion of residues from the C-terminal end, and, as shown in Fig. 3, this is associated with a concomitant decrease in receptor occupancy at pH 6.4. Taken together, these data suggest that His residues in the C-terminal region of the IgE molecule make a contribution toward the maintenance of the high affinity interaction between IgE and FcεRIα since this is largely determined by the slow rate of dissociation of the ligand from the receptor.

Results obtained in the current study differ in one significant respect from those in our previous investigation (6), where the FcεRI-blocking capacity of IgE-derived fragments was evaluated by the senior investigator, who performed passive cutaneous anaphylaxis tests in his own skin. Employing a well defined cellular assay system (23), we demonstrate here that N-terminal IgE sequences can be deleted beyond residue 340 without any significant effect on the kinetics of ligand/receptor interaction. Our data show that the essential determinant for hIgE/FcεRI recognition depends on a consecutive sequence comprising 11 amino acids computed to form a loop at the interface between the Cε3 and Cε4 domain (18). In accord with others (7–10), our observations exclude any direct contribution of Cε4-specific residues as proposed by Stanworth *et al.* (12). Our results confirm and extend those made by Nissim *et al.* (8–10), who demonstrated that the receptor binding site in IgE is located in the Cε3 domain. They differ from the claims of

Hamburger (29) and Gould *et al.* (20, 21), who propose, respectively, that residues 330–334 and 329–340 in the switch region between Cε2 and Cε3 are essential for IgE/FcεRI binding. As the results of our study clearly demonstrate, these sequences can be deleted without any major influence on the kinetics of hIgE/FcεRI interaction.

It is interesting to note that the active core sequence identified by us corresponds closely to the hIgE-derived peptide generated by Nio *et al.* (15), who report its capability to block the binding of antigen-specific IgE to cells expressing FcεRI at concentrations in the mM range (15).

Although the results of our study indicate that fragments containing the Cε2 domain show an increased susceptibility to proteolysis (Fig. 1C), the inclusion of the protease inhibitor phenylmethylsulfonyl fluoride during the isolation procedure facilitates the purification of peptides that engage FcεRI/II. Using our published method, others have been unable to generate ϵ -chain fragments in *E. coli* that retain FcεRI-binding capacity and have attributed this failure to folding problems (11). At least one other laboratory has expressed ϵ -chain fragments in *E. coli* which are biologically active (30).

Based on the outcome of FcεRI binding studies with chimeric and mutant hIgE molecules, Presta and co-workers (14) proposed that six amino acid residues located in three loops, C-D, E-F, and F-G, computed to form the outer ridge on the most exposed side of the Cε3 domain, are involved in receptor binding primarily by electrostatic interactions (14). These conclusions were based on the observation that replacement of these

residues reduced the binding of variant molecules to FcεRIα relative to native hIgE. We disagree with their conclusion in view of the fact that most of the mutations at Arg³⁷⁶ (408), Ser³⁷⁸ (411), Lys³⁸⁰ (415), Glu⁴¹⁴ (452), Arg⁴²⁷ (465), and Met⁴²⁹ (469) (Presta *et al.* (14) numbering scheme in parentheses), which affect IgE/FcεRI interaction to a greater or lesser extent, are invariably due to replacements by residues of opposite charge or by a Pro, changes which could cause structural rearrangements. In contrast, more conservative substitutions of these residues either have little effect or cause an apparent enhancement of binding (Ref. 14, Table I). Our own study shows that *e.g.* a single point mutation involving Cys³²⁸, which by itself is not required for either FcεRI or FcεRII binding (18), can have a dramatic effect on the conformation of the IgE molecule. Its substitution by Met, but not Ser, destroys binding to both receptors (18). When we compared the intrinsic fluorescence of Trp residues in the native and IgE Met³²⁸ molecule, we found that on average native IgE has 41% of its Trp residues exposed to solvent, while IgE Met³²⁸ was found to have only

22% of Trp residues exposed, although both molecules were recognized by a conformation-dependent monoclonal antibody directed against the Ce2 domain. This observation shows that the substitution of a single amino acid that is not involved in receptor recognition can induce a significant deformation in structure and profoundly affect ligand/receptor association.

Presta *et al.* (14) have also claimed that the grafting of loops C-D, E-F, and F-G and the inter-Ce2/3 switch region into hIgG (which they refer to as IgGEL), conferred FcεRI binding to hIgG₁. Their own data, however, on the binding of variant IgE do not support this interpretation. It is important to point out that their chimeric IgGEL construct still retains the endogenous IgG₁ loop A-B sequence, which when grafted into hIgE (Ref. 14, Table I, variant 1) effected a 97% decrease in binding.

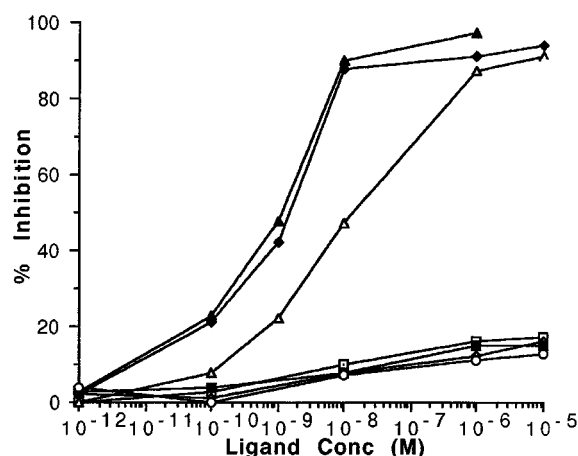


FIG. 2. Percentage of inhibition of ¹²⁵I-hIgE binding to RBL-2/C cells by native and recombinant hIgE-derived ε-chain fragments. To measure the inhibition (IC₅₀) of ¹²⁵I-hIgE to RBL-2/C cells by native IgE and recombinant ε-chain fragments, cells were preincubated at 22 °C for 1 h with increasing concentrations (10⁻¹²–10⁻⁵ M) of each of the unlabeled GST fusion peptides in 125 μl of binding buffer or, as a negative control, GST or binding buffer. ¹²⁵I-hIgE was then added (1 nM). After 45 min, the cells were washed twice with 0.5 ml of binding buffer and lysed in the same volume of lysis buffer, and aliquots were removed for γ-counting. The IC₅₀ values for GST·ε(226–547) and GST·ε(226–357) (data not shown) were identical to those observed for hIgE (▲) and GST·ε(226–354) (△). GST·ε(226–340) (◇) and GST·ε(355–547) (■), where the sequence common to all fragments that can engage FcεRI has been deleted by either the N- or C-terminal truncation, show inhibition levels similar to that obtained with GST (□) and all other fragments classified as nonbinders in Fig. 1. ♦, GST·ε(340–547); ○, GST·ε(440–547). Data shown represent the means of at least three separate experiments carried out in duplicate.

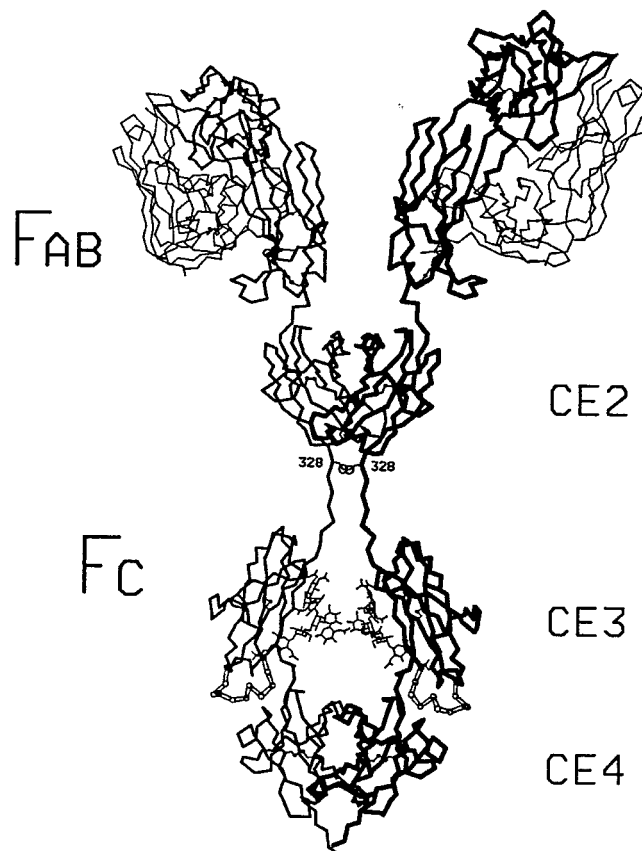


FIG. 3. Drawing of the α-carbon trace of a model structure (18, 19) for hIgE with various fragments and domains indicated. The light chains are drawn with thin lines and the heavy chains with thick lines, one thicker than the other. The interchain Cys at position 328 is labeled. The 11-amino acid segment 343–353, which is common to all IgE-derived peptides that bind to FcεRI, is drawn with bigger circles and wide, empty bonds in both heavy chains.

TABLE I
Binding of native and recombinant human and rat IgE-derived ε-chain fragments to hFcεRIα

The kinetics of association and dissociation of the affinity-purified ligands were determined at 22 °C as described under "Materials and Methods." Kinetic constants and IC₅₀, which represents the concentration at which 50% inhibition of ¹²⁵I-IgE V_{Nphs} binding was obtained, were calculated on the basis that GST·ε(226–547), GST·ε(326–547), and GST·ε(340–547) form dimers, while GST·ε(226–354) is a monomer. Nonbinders showed no binding above background at 10⁻⁵ M. (Data shown represent the means of *n* = number of experiments carried out in duplicate).

Ligand	<i>k</i> ₊₁ M ⁻¹ s ⁻¹	<i>k</i> ₋₁ s ⁻¹	<i>K</i> _d M	IC ₅₀ nM	<i>n</i>
hIgE	3.14 × 10 ⁵	1.3 × 10 ⁻⁵	4.1 × 10 ⁻¹¹	14	>8
hIgE·ε(341–356)	4.3 × 10 ⁴	1.7 × 10 ⁻⁵	3.9 × 10 ⁻¹⁰	ND ^a	1
GST·ε(226–547)	3.6 × 10 ⁵	1.6 × 10 ⁻⁵	4.4 × 10 ⁻¹¹	19	2
GST·ε(326–547)	4.1 × 10 ⁵	2.95 × 10 ⁻⁵	7.2 × 10 ⁻¹¹	23	2
GST·ε(340–547)	4.2 × 10 ⁵	9.8 × 10 ⁻⁵	2.3 × 10 ⁻¹⁰	34	2
GST·ε(226–354)	4.3 × 10 ⁵	6.02 × 10 ⁻³	1.4 × 10 ⁻⁸	332	3

^a ND, not determined.

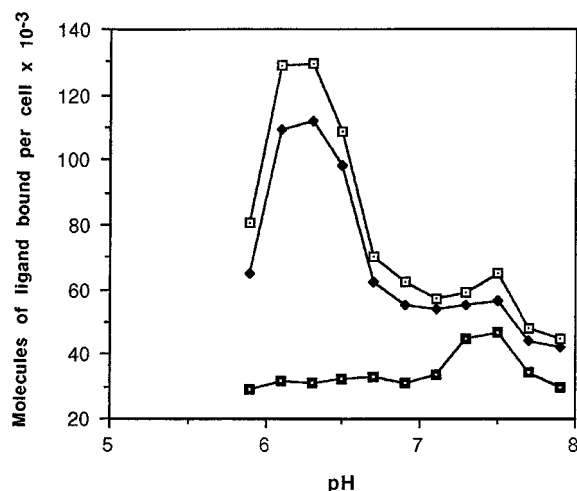


FIG. 4. pH profile for the binding of native IgE and recombinant IgE-derived peptides to RBL-2/2/C cells. α -chain-transfected RBL-2/2/C clones were distributed into 48-well plates at 10^5 cells/well and incubated with 10^{-6} M dexamethazone for 24 h at 37 °C. Prior to the assay cells were washed twice with 0.5 ml of saline containing 0.4% BSA and preincubated with 125 μ l of 50 mM phosphate-buffered saline containing 0.4% BSA (pH range 5.9–8.1) for 10 min at 37 °C before adding 50 μ l of 2 μ g of ^{125} I-labeled IgE (□), 0.7 μ g of GST- ϵ (340–547) (◆), and GST- ϵ (226–354) (■). Cells were incubated for 30 min, after which unbound ligand was removed by washing with binding buffer before measurement of cell-bound label. Results were corrected for nonspecific binding. (Data shown represent the means of two determinations carried out in duplicate)

This represents a greater reduction in activity than any other loop replacements described in their study. Data in their Fig. 3, which is interpreted by them to support their claim that the IgGEL chimera can recognize FcεRI, demonstrate the opposite since they show that when CHO 3D10 cells are incubated with IgGEL at a concentration of 1 μ g/ml, less than 2% of cells become labeled. Since these are the criteria that they applied for the assignment of the receptor binding activity of all other chimeric and mutant IgE molecules described in their investigation (Ref. 14, Table I), we conclude that IgGEL has the same affinity for FcεRI as variant 1, which is less active than any other chimera or mutant reported in their study. Although Fig. 3 of their paper (14) appears to suggest ~40% binding at 20 μ g/ml IgGEL, there is no evidence for saturation, and the slope of the line is indicative of nonspecific binding.

In our opinion, the data of Presta *et al.* provide compelling evidence that the A-B loop in hIgE comprises the essential structural determinant for IgE/FcεRI α interaction because the only difference between their variant 1 and native hIgE is that in variant 1 the core sequence, which we have shown to be common to all hIgE fragments that can engage hFcεRI α , is replaced by the IgG1 homologue.

As our study shows, sequences N- or C-terminal to this core peptide are necessary to provide structural scaffolding for the maintenance of a receptor binding conformation since the core peptide alone cannot engage the receptor. Deletion of C-terminal, but not N-terminal, sequences diminishes receptor occupancy at pH 6.4 and increases the dissociation of the ligand from the receptor. We conclude that residues, including His, in the C-terminal domain make an important contribution toward the maintenance of the high affinity of interaction between IgE and FcεRI α .

Although human and rodent IgE are highly homologous, rodent IgE can engage only hFcεRI but not hFcεRII (10). In contrast, hIgE cannot bind to either of the rodent receptors. The structural basis for this phenomenon is unknown. In the

current study we have replaced the human A loop by the rat homologue and find that the chimera still recognizes hFcεRI. This graft, however, does not confer binding to rFcεRI, indicating that conformational determinants outside the A loop in hIgE inhibit recognition of rFcεRI. The fact that this replacement destroys the binding of hIgE to hFcεRII indicates exquisite species specificity and suggests that residues in this motif contribute to the binding to both receptors. Nissim *et al.* (10) have demonstrated that the species specificity for recognition of FcεRII by murine and hIgE is contained in the Cε3 domain and proposed that part of the binding energy for hFcεRII interaction is contributed by amino acid residues between 346 and 356.

At present, limited structural information is available regarding the interaction between IgE and its receptors. Unlike IgG/FcγRn interaction, where the ligand can engage two receptors, IgE molecules bind to FcεRI α in a 1:1 stoichiometry, although bilateral symmetrical protection to proteolysis has been observed when rodent IgE is complexed to the α -chain (24, 31, 32). This has been explained in terms of a bent conformation of IgE (33), where the second ϵ -chain becomes inaccessible to an additional copy of the receptor, or to antibodies directed against epitopes in IgE that become masked following receptor engagement. There is little evidence for a beneficial role for IgE antibodies except in parasitic diseases, and such epitopes may therefore have an application as immunogens for the therapy of IgE-mediated allergies, since naturally occurring and monoclonal antibodies (33–36) have been described that block the binding of IgE to cells expressing FcεRI but do not trigger mediator release. An improved understanding of the docking of hIgE to its receptors will provide the structural information needed for the rational design of such immunogens. The identification of the binding site constitutes a major step in this direction.

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